Dental Research Journal

Original Article

Cytotoxicity evaluation of three resin-based sealers on an L929 cell line

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Received: January 2012
Accepted: June 2012

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ABSTRACT

Background: Endodontic sealers usually come in contact with adjacent tissues and their biocompatibility is key in a successful treatment. The purpose of this study was to assess the cytotoxicity of three resin-based sealers, namely AH Plus, EndoREZ, and Epiphany in set and fresh states on an L929 cell line.

Materials and Methods: In this *in vitro* experimental study, the materials were mixed according to the manufacturers' instructions, and were divided into two groups, fresh and set. The elutes of materials were prepared separately and were incubated with L929 fibroblasts for 1 hour, 24 hours, and 72 hours. Pulp Canal Sealer and Dulbecco's Modified Eagle Medium (DMEM) served as positive and negative controls respectively. Cell viability was evaluated by MTT assay ([3-4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide succinate), after 1 hour, 24 hours, and 72 hours. The data were analyzed by analysis of variance (ANOVA), and Tukey multiple comparison test.

Results: After 1 hour, fresh Epiphany and fresh AH Plus were significantly more cytotoxic than their set samples. No significant difference was perceived between cytotoxicity of fresh state of sealers and positive control, or between set state and negative control. After 24 hours, both fresh and set samples of all materials were significantly more cytotoxic than the negative control group, and were less cytotoxic than the positive control group. After 72 hours, the fresh and set samples of all materials were as cytotoxic as the positive control group. At each time point, no significant difference was perceived among different materials in terms of cell viability.

Conclusion: The observed differences among the cytotoxicity of AH Plus, EndoREZ, and Epiphany did not reach a significant level at comparable time points after exposure.

Key Words: Cytotoxicity, fibroblasts, MTT assay, root canal sealer

INTRODUCTION

Sealers are usually not confined within the root canal, and so, root canal sealing materials usually come in contact with adjacent tissues.[1,2] In fact, some exposure of periradicular tissues to sealing materials, through several connections, such as dentinal tubules, accessory and lateral canals, and apical foramina, is inevitable. Studies have also reported that elutable substances and degradation or corrosion products from root canal fillings might gain access to adjacent tissues.[3,4] Subsequently, if sealers are of toxic materials which could cause periapical tissue injury, the outcome may be compromised. The biocompatibility of sealants is key in a successful treatment.[3,5,6] Several factors, including setting characteristics, composition, leachable components, stability of set sealers, and the contact area between the sealer and surrounding tissue, affect the biocompatibility of root canal sealers.[5,7]

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Different compositions of sealers are generally used in endodontics. Such compositions are based on a variety of materials, ranging from zinc oxide eugenol, calcium hydroxide, and mineral trioxide aggregates to glass-ionomer or polymers such as epoxy resins polydimethylsiloxane and methacrylates.[3] An increasing interest in using resin-based sealers has emerged in recent years, mainly because of their improved bonding to root dentin.

The aim of this *in vitro* study was to assess the cytotoxicity of AHPlus (an epoxy resin-based sealer), EndoREZ (a single-methacrylate-based sealer), and Epiphany (a multi-methacrylate resin-based sealer) in set and fresh states by the MTT assay ([3-4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide succinate), which tests for mitochondrial enzyme activity.[2]

MATERIALS AND METHODS

Preparation of elutes of endodontic sealers

The endodontic sealers investigated in this *in vitro* experimental study were AH Plus (Dentsply, De Trey GmbH, Konstanz, Germany), EndoREZ (Ultradent Products Inc., South Jordan, UT, USA), and Epiphany (Pentron Clinical Technologies, LLCC, Wallingford, CT, USA). The sealers were prepared under aseptic conditions according to the manufacturers' instructions. Then, they were divided into two groups:

Set group

One gram of each of materials was formed into small disks of same size (each disk approximately 50 mg), and then the disks were dispensed into one well of a six-well tissue culture plate. The sealer disks of EndoREZ and Epiphany were light cured for 40s using a light-curing unit at 780 mW/cm^2 from one side. All three sealers were then maintained for 24 hours at 37°C in a humid atmosphere of 5% CO_2 under sterile conditions, so that the polymerization went to completion.

Fresh group

The sealer disks dispensed immediately after mixing into the wells of a six-well tissue culture plate. No light curing or incubation was performed on the disks in this group.

In order to perform the extraction, each specimen was stored in an incubator for 24 hours, in 10 ml of cell culture medium at 37°C in a humid atmosphere of 5% CO_2.

After 24 hours, the plates were removed from the incubator, and the elute was filtered (filters: Pore size µm 0/22, Schleicher and Schuell, Dassel, Germany). These elutes were then used for cytotoxicity testing by the MTT assay.

Cytotoxicity testing

Mouse L929 fibroblasts from passage 4 were seeded into three 96-well tissue culture plates at a concentration of 10^5 cells per well. Each group was incubated in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco, USA), 100 U/ml Penicillin, and 100 µg/ml Streptomycin (Sigma-Aldrich, St. Louis, MO, USA), for 24 hours at 37°C, 5% CO_2 and 98% humidity. Each of three plates was devoted to a specific time point (1 hour, 24 hours, and 72 hours) and 30 wells of a tissue culture plate were assigned to each material in each time point.

The elutes of materials were tested for cytotoxicity in contact with L929 fibroblasts. Culture mediums were removed and 200 µl of elutes were dispensed into each well. The plates were incubated at 37°C, 5% CO_2 and 98% humidity for 1, 24, and 72 hours, and then were evaluated by MTT assay. Pulp Canal Sealer (SybronEndo Corporation, Orange, CA, USA) disks and DMEM served as positive and negative controls respectively. Pulp Canal Sealer was used as the positive control because of its known *in vitro* cytotoxicity.[8,9]

MTT assay

The MTT assay was carried out in a sterile area. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Merk, Darmstadt, Germany) was added in 1:10 ratio of the culture media volume. Extract was removed from wells, and 150 µl of MTT solution was added to each well. Then, the plates were incubated for 4 hours at 37°C at 95% humidity and 5% CO_2. After that, 100 µl of 0.04 mol/l HCl in isopropanol was used to dissolve the crystals of formazan. The substances in the wells of tissue culture plate were smoothly mixed so that the crystals were dissolved completely. The absorbance was computed at a wavelength of 570 nm using an ELISA plate reader (Anthos 2020, Anthos Labtec Salzburg, Austria).[10]

Data and statistical analysis

Cell viability was used to measure the toxicity of the endodontic sealers using MTT assay. The data were analyzed by analysis of variance (ANOVA), and

follow-up comparisons between groups were made using the Tukey multiple comparison test (at a 95% confidence interval level; $\alpha = 0.05$). The statistical significance level was established at $P < 0.05$.

RESULTS

The optical density of samples (mean ± SD) after exposure to the elutes of the root canal sealers is shown in Table 1.

After 1 hour, fresh Epiphany and fresh AH Plus were found to be significantly more cytotoxic than their set samples ($P < 0.05$). No significant difference was perceived between cytotoxicity of fresh state of sealers and positive control, or between set state and negative control. After 24 hours, both fresh and set samples of all materials were significantly more cytotoxic than the negative control group, and were less cytotoxic than the positive control group ($P < 0.05$). After 72 hours, the fresh and set samples of all materials were as cytotoxic as the positive control group. At each time point, no significant difference in cell viability was perceived among different materials [Table 1].

Fresh samples of each material were significantly more cytotoxic at 1-hour or 72-hour readings than at 24-hour reading. In regard to set samples, each material was more cytotoxic at 72-hour reading than at 1-hour and 24-hour readings. In addition, the cytotoxicity of set EndoREZ and set AH Plus reached significantly higher levels at 1-hour reading than at 24-hour reading ($P < 0.05$).

DISCUSSION

If endodontic sealers come in contact with the periapical tissues, any cytotoxic degradation product may gain access to, and damage surrounding tissues. As a result, it is necessary that root canal sealers be biocompatible.[11] In this regard, every sealer must be tested biologically through *in vitro* and *in vivo* experiments before any clinical use.[8] While the *in vitro* test results can provide a cytotoxicity ranking for materials, it is crucial that sealers be tested by *in vitro* tests.[12] The purpose of this study was to evaluate the toxicity of elutes of several root canal sealers on fibroblasts, which are an ISO-approved cell type.[13] Cytotoxicity was determined by the MTT assay, a common method for the evaluation of biocompatibility of endodontic sealers.[4,9,14] MTT assay determines the cytotoxicity by evaluating the ability of mitochondrial dehydrogenase enzymes in converting the yellow water-soluble tetrazolium salt MTT into dark blue formazan crystals. Although this method has the limitation of its *in vitro* nature and so its clinical validity is not guaranteed, it has several advantageous such as its simplicity, rapidity, and accuracy. It also does not require radioisotopes.[15] In this study, the materials were used both in fresh and set states in order to investigate the effect of setting process in cytotoxicity of experimental material as it has stated in former studies such as lodiene *et al*.[16] and merdad *et al*.[17]

Results of this study show that fresh samples of all three materials were cytotoxic at 1 hour following exposure. Minute release of formaldehyde has been recognized as the cause of cytotoxicity of AH Plus.[18] In addition, the cytotoxicity of EndoREZ has been attributed to the presence of urethane dimethacrylate (UDMA), a known toxic agent, in the structure of this sealer.[19] As for Epiphany, the cytotoxicity might be explained by the high resin content of this sealer, resins which consist of bisphenol A-glycidyl methacrylate (Bis-GMA), ethoxylated Bis-GMA, UDMA, and hydrophilic difunctional methacrylates.[20,21] It is also possible that degradation causes leaching of monomers and filler particles, resulting in cytotoxicity of this sealer.[21,22] Our results about the cytotoxicity of AH Plus, EndoREZ, and Epiphany, are in general agreement with the majority of studies, which have also reported cytotoxic characteristic of these materials.[4,14,21-24]

One hour after exposure, the set groups were proved to be significantly more biocompatible than the fresh groups, a finding that is in agreement with the study of Lodiene *et al*.[16] This reduction of cytotoxicity

Table 1: Optical density of samples (mean ± SD) at 1 hour, 24 hours, and 72 hours after exposure to the elutes of the root canal sealers

Materials	1 hour	24 hours	72 hours
AHplus			
Fresh	0.0130±0.0043	0.0830±0.0186	0.0130±0.0143
Set	0.0724±0.0237	0.1036±0.0052	0.0174±0.0196
Endorez			
Fresh	0.0318±0.0054	0.0818±0.0137	0.0152±0.0186
Set	0.0634±0.0157	0.1030±0.0159	0.0146±0.0139
Epiphany			
Fresh	0.0021±0.0020	0.1056±0.0087	0.0066±0.0046
Set	0.0790±0.0234	0.1210±0.0130	0.0126±0.0120
Control –	0.0792±0.0215	0.5822±0.0443	0.2408±0.0474
Control +	0.0020±0.0011	0.0022±0.0013	0.0028±0.0008

after setting might have occurred because of a decrease in formaldehyde release in AH Plus,[14,17,25] or because of conversion of monomers into polymers in the setting process.[26] Though the difference between fresh and set state of EndoREZ was not significantly different. This result indicates that, in comparison with properties of other sealers, cytotoxic properties of EndoREZ are less affected by setting interaction, and that this material releases more toxic agents after a setting period.

Similar to some previous studies,[7,16,17] our results showed that all materials, in both set and fresh states, were more biocompatible at 24 hours after exposure than at 1 hour after; however, in the Epiphany case, this difference did not reach a significant level. Such a phenomenon might have occurred because the setting process had not completed, considering studies which have reported that complete setting of Epiphany could last for even 7 days.[27]

Despite the common assumption that toxicity decreases over time,[14,17,22] results of our study show that after 72 hours, cell viability values reduce for all samples. Some previous studies, in agreement with our study, have reported a decrease in cell viability values for AH Plus in this reading point.[14,28] The reduction in cell viability values may be attributed to the decrease in nutrients in the culture medium, or to an accumulation of toxic agents in the medium. The perceived decrease in the cell viability of the negative control group (including DMEM without any elute) also stands in agreement with the above theory.

For each material, no significant difference was observed among the cytotoxicity at 1 hour, 24 hours, and 72 hours after exposure reading points. Our results are in agreement with results of Bouillaguet *et al*.[28] and Camargo *et al*.,[12] who have reported that Epiphany and AH Plus exhibit similar toxicity in relation to cell survival. Previous studies have reported a variety of cytotoxicity for these materials. Those studies have reported contradictory results in comparing cytotoxicity of these materials.[29-33] These contradictory results might be attributed to experimental conditions, different reading points, target cell type, cell material contact method, preparation of extracts or solid specimens and exposure time.[34]

CONCLUSION

The observed differences among the cytotoxicity of AH Plus, EndoREZ, and Epiphany did not reach a significant level at comparable time points after exposure. Furthermore, the setting procedure of the experimental endodontic sealers reduced their cytotoxicity early after exposure but did not affect that in afterward. Additional *in vivo* studies are proposed to confirm these *ex vivo* results.

ACKNOWLEDGEMENT

This article was based on postgraduate thesis by Nima Moradi Majd which was successfully completed under supervision of Dr Hengameh Ashraf by Dental School of Shahid Beheshti University of Medical Sciences.

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How to cite this article: Ashraf H, MoradiMajd N, Mozayeni MA, Dianat O, Mahjour F, Yadegari Z. Cytotoxicity evaluation of three resin-based sealers on an L929 cell line. Dent Res J 2012;9:549-53.

Source of Support: Nil. **Conflict of Interest:** None declared.